

Screening of Antibacterial Activity of Six Plant Essential Oils Against Pathogenic Bacterial Strains

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Abstract: In the present investigation antimicrobial activity of six different plant essential oils i.e., citrus (*Citrus lemon*), olive (*Olea europaea*), ajwain (*Trachyspermum ammi*), almond (*Amygdalus communis*), Bavchi (*Psoralea corylifolia*) and neem (*Azadirachta indica*) oils have been evaluated. After bioassays, most of the essential oils were found susceptible to both Gram-positive bacteria such as *Lactobacillus acidophilus*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus* and Gram-negative bacteria *Klebsiella pneumoniae*, *Escherichia coli*. For screening of antimicrobial susceptibility in each essential oil, both positive and negative controls were set to determine MIC (minimum inhibitory concentration), MBC values (minimum bactericidal concentration) and growth inhibition zone diameters. Among all essential oils almond and neem oils were found to be highly bactericidal, as it has shown lowest MIC and MBC values and high growth inhibition zone diameter in comparison to antibiotics. Present study reveals significantly higher broad-spectrum antibacterial activity in essential oils than antibiotics i.e., tetracycline, ampicillin and ciprofloxacin.

Key words: MIC, MBC, antibacterial activity, essential oils

INTRODUCTION

In the present time, drug resistance in microbes is a very serious problem. Hence, plant origin herbal medicines are considered as safe alternatives of synthetic drugs. There are varied methods of medicines like Ayurveda, Homeopathy and Unani, which utilize plant materials for drug production. Currently, Ayurveda considered as a vital system of medicine and governed the worldwide recognition and having non-toxic substances. However, newly discovered non-antibiotic substances such as certain essential oils (Sonboli *et al.*, 2006) and their constituent chemicals (Chavan *et al.*, 2006) have shown good fighting potential against drug resistant pathogens (Cowan, 1999; Ahmad and Beg, 2001). Essential oils are aromatic oily liquids, which are obtained from various plant parts such as flowers, buds, seeds, leaves, twigs, bark, woods, fruits and roots by steam distillation. Scientifically these oils have been proved highly potent antimicrobial agents in comparison to antibiotics. These plant essential oils are rich source of scents and used in food preservation and aromatherapy. These possess multiple antimicrobial i.e., antibacterial (Ozcan *et al.*, 2006), antifungal (Cafarchia *et al.*, 2002), anticancer, antiviral and antioxidant properties (Salehi *et al.*, 2005; Vardar-Unlu *et al.*, 2003), against viruses, bacteria and fungi (Kalemba and Kunicka, 2003). Some essential oils such as aniseed, calms, camphor, cedar-wood, cinnamon, eucalyptus, geranium, lavender,

lemon, lemongrass, lime, mint, nutmeg, rosemary, basil, vetiver and winter green are traditionally used by people in different parts of the world. Cinnamon (Prabuseenivasn *et al.*, 2006), clove, rosemary and lavender oils have shown both antibacterial and antifungal properties (Quale *et al.*, 1996; Chang *et al.*, 2001; Wilkinson and Cavanagh, 2005). Besides this, Cinnamon oil possesses anti-diabetic and anti-inflammatory activity (Mittra *et al.*, 2000), while lemon, rosemary and peppermint exhibit anticancer activities (Imai *et al.*, 2001). In the present study antimicrobial potential of six different plant essential oils was screened against seven pathogenic bacterial strains i.e., *Klebsiella pneumoniae*, *E. coli*, *Bacillus cerues*, *Staphylococcus aureus*, *Lactobacillus acidophilus*, *Micrococcus luteus* and *Streptococcus pneumoniae* in various antibacterial bioassays. For antimicrobial susceptibility of each essential oil MIC and MBC values and growth inhibition zone diameters were determined.

MATERIALS AND METHODS

This study was conducted from 25 February to 30 March, 2010 in at DDU Gorakhpur University Gorakhpur (UP) India

Extraction of essential oils: Essential oils used in this study were extracted from the spices and other plant material. Spices were purchased from the standard spice

supplying herbal companies. These were separately grounded and powdered in domestic Mixi and hydro distilled in a Clevenger's apparatus by the technique of Guenther (1948) to obtained essential oils. Before application, solubility of all essential oil was tested. Dilution was made after a known volume of each oil was diluted by adding fresh solvents and stored at 5°C till used.

Bacterial cultures: Cultures of seven pathogenic bacterial strains each of *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 11778), *Lactobacillus acidophilus* (ATCC 53103), *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae*, (ATCC 15380) and *Streptococcus pneumoniae* (ATCC 12755) bacterial culture were maintained in Luria Broth (2% w/v) a constant at 37°C in the laboratory. For inoculation, a portion (100 µl) of overnight culture of each bacterial strain was mixed in 15 ml of media for each test and control separately. For activity testing, bacterial cultures were stored at 4°C and sub cultured after every 8th day in solid agar plates

Screening of antibacterial activity: Antimicrobial activity of essential oils on bacterial growth was accessed in presence of different increasing concentrations of essential oils. For this purpose, essential oils were diluted by using serial micro dilution method with Luria Broth culture medium at a final concentration range from 32 to 0.0078 µl/mL. In each test essential oils were added to fresh suspension after making serial dilution up to 10^{-10} . Each and every essential oil was assayed for antibacterial activity in triplicate. Before conducting experiments all the conditions were standardized to determine MIC and MBC values *in vitro*.

Filter paper disc diffusion assay: Agar disc diffusion method was used for screening of antimicrobial activity of each essential oil. For antimicrobial activity testing essential oils were diluted by adding equal volume of solvent. From this a known volume i.e., 2-32 mL of each essential oil was coated on separate sterile filter paper discs (Whatman No. 1) measuring 6 mm in size. These oil-impregnated discs were made dry under laminar flow cabinet. Bacterial inoculum was spread evenly on to the surface of each agar plate with sterile rubber pad spreader and essential oil coated discs were positioned in the centre of inoculated agar plate. Each essential oil was assayed in triplicate. Sterile distilled water was used as negative control, while broad-spectrum antibiotics i.e. tetracycline, ampicillin and ciprofloxacin were used as positive control for obtaining comparative results. All treated and untreated plates were incubated for 24 h at 37 °C and size of inhibition zone diameters surrounding filter paper disc was measured.

For determination of Minimum Bactericidal Concentration (MBC) growth inhibitory assays were

performed. For this purpose, inoculum size was adjusted to prepare a final colony number as 10^8 colony forming units (CFU/ml) in sterile agar plates. Both test and control cultures were kept at 37°C for 24 h. For comparison, both negative and positive controls were set and bacterial colony number was counted. The least concentration at which no visible growth was obtained in agar plates was considered as MBC. For evaluation of inhibition two parallel controls were set and bacterial growth was obtained in presence and absence of various quantities of essential oils.

Statistical analysis: The results were interpreted with the standard deviation. Student t-test was applied to know significant differences between the antimicrobial effectiveness of each oil and antibiotics. The data was also statistically analyzed by applying ANOVA to know the significant difference in antimicrobial susceptibility of broad-spectrum antibiotics, and various oils. The related effectiveness was also tested by applying linear correlation between control and tests.

RESULTS

Determination of MIC and MBC values: The MIC values of six different essential oils are presented in Table 1. Each essential oil has shown very high susceptibility against bacterial strains, as MIC values obtained were very low. The MIC value of citrus oil was obtained lowest against *B. cereus* (0.25 µl), while it was obtained 1.0 µl/mL against *Lactobacillus acidophilus*, *Klebsiella pneumoniae*, (Table 1). MIC value in olive oil was obtained lowest 0.50 µl against *E. coli*, and *Lactobacillus*, while highest (2 µl) in case of *Micrococcus luteus*. Olive oil has shown intermediate effect against *Staphylococcus aureus* and *Micrococcus luteus* and *Streptococcus pneumoniae*. Ajwaine oil was proved highly lethal to *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Lactobacillus acidophilus* with lowest MIC value i.e., 0.25 µl (Table 1). Almond oil has shown lowest MIC value i.e., 0.5 µl/mL for *E. coli* and *B. cereus*, while bavchi oil was found more bactericidal to all different bacterial strains as it has shown MIC value in a range of 0.5 to 1 µl/mL. Neem oil has shown better effect against *Streptococcus pneumoniae*, *B. cereus* and *Lactobacillus acidophilus* and MIC values obtained was 0.5 µl separately (Table 1). Similarly, MBC values of each essential oil were also determined in bacterial cultures, which were found to be higher against *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Streptococcus pneumoniae* and *B. cereus* (0.25-2 µl), while the lower MBC values of essential oils were obtained against *B. cereus*, *Lactobacillus* and *E. coli* in comparison to *Micrococcus luteus* and *Klebsiella pneumoniae* (Table 2).

Table 1: Determination of MIC value ($\mu\text{L/mL}$) of six essential oils against pathogenic bacterial strains

Essential oils in $\mu\text{L/mL}$	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>M. luteus</i>	<i>S. pneumoniae</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>L. acidophilus</i>
Citrus	0.25	0.125	1.0	1.0	2.0	0.125	0.25
Clove	4.0	0.5	2.0	0.125	0.25	2.0	0.25
Ajwain	1.0	0.125	2.0	0.125	4.0	0.25	0.125
Almond	1.0	0.25	0.5	0.5	1.0	0.25	0.5
Bavchi	0.25	1.0	0.25	0.5	0.125	0.5	0.125
Neem	2.0	2.0	2.0	0.5	4.0	0.25	0.125
Tetracycline	0.892	0.892	0.446	0.446	0.892	0.892	0.892
Ampicillin	0.223	0.446	0.892	0.223	0.446	0.446	0.223
Ciprofloxacin	0.892	0.446	0.223	0.892	0.446	0.892	0.223

Table 2: Determination of MBC values ($\mu\text{L/mL}$) of six essential oils against pathogenic bacterial strains

Essential oils in $\mu\text{L/mL}$	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>M. luteus</i>	<i>S. pneumoniae</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>L. acidophilus</i>
Citrus	0.5	2.0	4.0	2.0	4.0	0.25	1.0
Clove	2.0	1.0	4.0	2.0	2.0	4.0	0.5
Ajwain	2.0	1.0	4.0	0.5	1.0	1.0	0.25
Almond	2.0	1.0	1.0	1.0	4.0	2.0	2.0
Bavchi	1.0	2.0	2.0	2.0	2.0	1.0	0.25
Neem	1.0	1.0	2.0	0.25	2.0	1.0	0.25
Tetracycline	0.915	0.915	7.32	0.982	0.915	0.915	0.915
Ampicillin	1.83	1.83	7.32	0.915	0.915	1.83	0.915
Ciprofloxacin	1.83	1.83	0.915	0.915	0.915	1.83	3.66

Table 3: The quantitation of microbial activity of different essential oils was measured by using agar disc diffusion method. The effectiveness of different essential oils is demonstrated by the size of the micro-organism growth inhibition zone around the filter paper disc, which is typically expressed as the diameter of the zone in mm*

Essential oil	volume used (μL)	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>M. luteus</i>	<i>S. pneumoniae</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>L. acidophilus</i>
Citrus	32	16.03 \pm 0.12	19.96 \pm 0.08	28.63 \pm 0.23	27.66 \pm 0.24	23.10 \pm 0.25	41.3 \pm 0.26	19.16 \pm 0.185
Olive	32	20.3 \pm 0.26	23.13 \pm 0.12	27.1 \pm 0.15	17.03 \pm 0.14	20.1 \pm 0.35	35.7 \pm 0.11	19.03 \pm 0.48
Ajwain	32	19.0 \pm 0.08	23.0 \pm 0.152	2.2 \pm 0.11	22.53 \pm 0.14	21.13 \pm 0.35	23.16 \pm 0.23	15.16 \pm 0.23
Almond	32	18.0 \pm 0.11	16.66 \pm 0.21	22.6 \pm 0.5	16.73 \pm 0.22	17.8 \pm 0.31	28.0 \pm 0.3	21.0 \pm 0.35
Bavchi	32	19.1 \pm 0.18	15.8 \pm 0.43	23.46 \pm 0.26	23.5 \pm 0.05	15.86 \pm 0.43	21.36 \pm 0.23	22.86 \pm 0.17
Neem	32	24.0 \pm 0.34	19.03 \pm 0.47	24.4 \pm 0.41	20.33 \pm 0.5	23.13 \pm 0.24	45.63 \pm 0.23	16.28 \pm 0.11
Tetracycline	8.0	24.66 \pm 0.28	25.23 \pm 0.23	24.1 \pm 0.23	23.43 \pm 0.24	19.73 \pm 0.405	19.06 \pm 0.08	24.0 \pm 0.305
Ampicillin	8.0	19.63 \pm 0.384	18.13 \pm 0.37	20.2 \pm 0.152	23.53 \pm 0.145	15.2 \pm 0.08	14.73 \pm 0.29	16.03 \pm 0.12
Ciprofloxacin	8.0	22.73 \pm 0.145	23.0 \pm 0.08	24.33 \pm 0.115	24.33 \pm 0.20	24.2 \pm 0.12	23.03 \pm 0.12	26.2 \pm 0.152

Control no antibiotic. *: The strength of activity is presented as resistant ($>7\text{mm}$), intermediate ($>12\text{mm}$) and susceptible ($>18\text{mm}$)

Inhibition zone diameter: In the present study, effectiveness of essential oils was also confirmed by filter paper disc diffusion assay and growth inhibitions zone diameters were measured in presence and absence of each essential oil. Results are presented Table 3. Citrus, Olive, Ajwain, Neem, and Almond oil have shown larger growth inhibition zone diameters in comparison to synthetic antibiotics (Plate 1 and 2). Citrus oil has shown 41 mm inhibition zone diameter against *B. cereus*, olive oil 35 mm against *B. cereus*, Ajwain oil 23 mm against both *E. coli* and *B. cereus*. Almond oil has shown 38 mm against *B. cereus* while Bavchi oil 23 mm against *Lactobacillus acidophilus*, *Streptococcus pneumoniae* and *Micrococcus luteus*. Neem oil has shown highest inhibition zone diameter i.e., 45 mm against *B. cereus* (Table 3). From the experiments, both olive and neem oils were found highly susceptible to *B. cereus* as growth inhibition zone diameters were obtained in a range 41-45 mm in size.

DISCUSSION

Present study reveals the antimicrobial susceptibility of essential oils against seven bacteria *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Lactobacillus acidophilus*, *Micrococcus luteus*, *B. cereus*, *Klebsiella pneumoniae* and *Escherichia coli*. It is proved by low

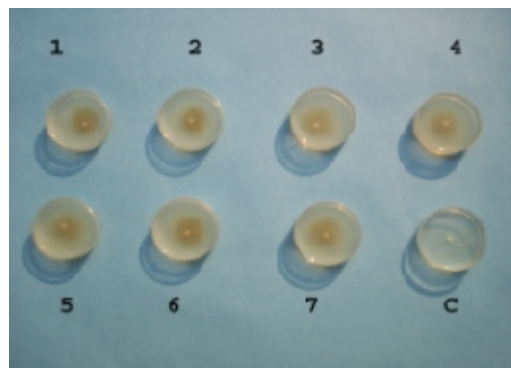


Plate 1: Agar disc diffusion test of Bavchi (*Psoralea corylifolia*) oil coated on 6 mm size Whatmann filter paper disc

MIC and MBC values obtained in essential oils when used against each bacterial culture. Among all essential oils both citrus and neem oil have shown lowest MIC values (0.125 $\mu\text{L/mL}$) against *E. coli* and *B. cereus* while olive oil has shown 0.5 $\mu\text{L/mL}$ MIC value against *E. coli* and *Lactobacillus acidophilus* (Table 1). Both ajwain and Bavchi oil have shown anti-microbial activity against *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Lactobacillus acidophilus* at a 0.25-0.5 $\mu\text{L/mL}$ concentration. Besides this, both Ajwain and Neem oil

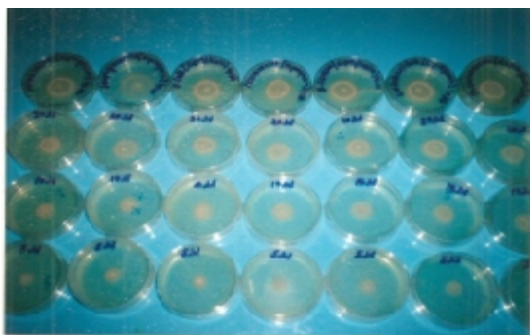


Plate 2: Agar disc diffusion test of Standard antibiotic drug (Ciprofloxacin)

were found highly bactericidal to *Streptococcus pneumoniae*, which was proved by low MBC values obtained i.e. in a range of 0.25-0.50 $\mu\text{L}/\text{mL}$ (Table 2). More specifically all essential oils tested have shown higher MBC values than MIC values against each bacterial strain. MBC value was found to be lowest in citrus oil (0.25 $\mu\text{L}/\text{mL}$) against *B. cereus* (Table 2). In similar bioassays, almond oil has shown stronger antimicrobial activity against *E. coli* than *Micrococcus luteus* and *Streptococcus pneumoniae*. It has shown 1.0 $\mu\text{L}/\text{mL}$ MBC against *Micrococcus luteus* (ATCC 9341). Essential oils have shown maximum susceptibility against *Lactobacillus acidophilus* bacteria at a very low MBC value i.e., 0.25-1.0 $\mu\text{L}/\text{mL}$. Finally, essential oils have shown significantly very low MIC and MBC values in comparison to broad-spectrum antibiotic drugs. This suggests that the essential oils are highly bactericidal.

Similar MIC values are also reported in juniper oil (70% v/v) Pepeljnjak *et al.*, 2005) against Gram +ve and Gram -ve bacteria. It has also shown stronger fungicidal activity against *Candida* sp. (MIC from 0.78 to 2% v/v). Similarly garlic oil, garlic powder and diallyl sulfur components have shown MIC and MBC values in a range of 8 to 32 $\mu\text{L}/\text{mL}$ and 16 to 32 $\mu\text{L}/\text{mL}$ against *Helicobacter pylori* (Gara *et al.*, 2000). Few essential oils obtained from different *Mentha* species i.e. *Mentha longifolia* L., *Mentha aquatica* and *Mentha piperita* L. (Mimica-Dukic *et al.*, 2003), *Hypericum scabrum*, *Hypericum scabroides* and *Hypericum triquetrifolium* exhibited broad-spectrum antibacterial activity against disease pathogens (Kizil *et al.*, 2004). Similarly *Dracocephalum foetidum* essential oil also exhibited strong antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) at a very low MIC value i.e., 26-2592 $\mu\text{L}/\text{mL}$ (Lee *et al.*, 2007). Besides essential oils, oil constituents such as allicin and diallyl sulfur (Gara *et al.*, 2000), luteolin (Brenes *et al.*, 1999), thymus (Shin and Kim, 2005), phenolics (Eduardo-Medina *et al.*, 2006) and Cavacrol (Burt *et al.*, 2005) isolated from various essential oils have also shown stronger antimicrobial

activity against few bacteria. Olive oil and its phenolic constituents showed antimicrobial activity against food borne pathogens such as *Helicobacter pylori* (Romero *et al.*, 2007). More over, di-terpenoids isolated from *Sagittaria pygmaea* have shown antibacterial activity against *Streptococcus mutans* (ATCC 25175) with MIC value of 15.6 $\mu\text{L}/\text{mL}$ (Liu *et al.*, 2007). More specifically, essential oils (Karaman *et al.*, 2001) obtained from *Foeniculum vulgare*, *Strithum mantimum* (Ruberto *et al.*, 2000), *Scoorodophelous zenkeri* (Kouokam *et al.*, 2002) and *Satureja* sp. (Azaz *et al.*, 2002), *Artemisia asiatica* (Kalembe *et al.*, 2002), *Mentha piperita* (Iskan *et al.*, 2002; Mimica-Dukic *et al.*, 2003), *Thymus aquaticus* (Faleiro *et al.*, 2003), *Achillea millefolium* (Candan *et al.*, 2003), *Coridothymus capitatus* (Goren *et al.*, 2003), *Scutellaria barbata* (Yu *et al.*, 2004), *Achillea ligustica* (Tuberoso *et al.*, 2005), *Thymbra spicata* (Kilic, 2006) *Cuminum cyminum* and *Carum carvi* (Iacobellis *et al.*, 2005), *Coriandrum sativum* and *Foeniculum vulgare* (Cantore *et al.*, 2004) and *Pimenta racemosa* var. *Trebinthina* (Sanez *et al.*, 2004) were found more potent against various pathogenic bacterial strains (Friedman *et al.*, 2004). Similar antimicrobial activity is reported in essential oils isolated from Amazonian basil, *Ocimum gratissimum* (Nakamura *et al.*, 1999) *Ocimum micranthum* (Sacchetti *et al.*, 2004), *Sesuvium portulacastrum* (Magwa *et al.*, 2006) and citrus peel oil (Lan Phi *et al.*, 2006).

Further effectiveness of each essential oil was determined by agar disc diffusion method and inhibition zone diameters were measured in presence and absence of each essential oil. (Plate 1 and 2). Based on growth inhibition zone diameters obtained bacterial strains were divided in to three categories i.e. resistant (>7 mm), intermediate (>12 mm), and susceptible (>18 mm). As in the present study, inhibition zone diameters were obtained more than 18mm in size, which are significantly ($p < 0.05$) much larger than the antibiotic drugs and proves susceptibility of essential oil. Similar growth inhibition zone diameters were also obtained by Prabuseenivasn *et al.* (2006) in different bacterial strains such as *Pseudomonas aeruginosa* 33.3 mm, *B. subtilis* 29.9 mm *P. vulgaris* 29.4 mm, *K. pneumoniae* 20.8 mm and *S. aureus*. Besides this, some plant essential oils have shown growth inhibitory effects against *Clostridium ferfringens*, *E. coli* and *Lactobacillus acidophilus* (Eduardo-Medina *et al.*, 2006), *Bacillus species* (Ozcan *et al.*, 2006), *Staphylococcus aureus* (Brady *et al.*, 2006; Ferrini *et al.*, 2006), *Solmonella enteritidis* (Raybaudi-Massilia *et al.*, 2006).

In the present study, essential oils have shown nearly equal antimicrobial effects on both gram positive and gram-negative bacteria in suspension culture. Olive, citrus and neem oils were found to be the most effective.

However, inhibition zone diameters obtained in filter paper disc diffusion assays have shown better effectiveness of essential oils against Gram-positive bacteria. It may be due to volatile action of essential oils and due to absence of lipo-polysaccharide layer in Gram-positive bacteria that might function as an effective barrier against any incoming bio-molecule (Inouye *et al.*, 2001; Delaquis *et al.*, 2002). There might be another possibility that essential oils may successfully inhibit microbial respiration and increase the plasma membrane permeability, which results in to death of bacterial cells after massive ion leakage (Lambert *et al.*, 2001; Walsh *et al.*, 2003). It may also happen due to hydrophilic nature of bacterial cell wall (Knobloch *et al.*, 1986). In the present study, almost all essential oils tested have shown strong antibacterial potential against pathogenic bacteria. After bioassays essential oils were found susceptible to both Gram-positive and Gram-negative bacteria.

CONCLUSION

This study emphasizes antimicrobial properties of plant essential oils against human pathogenic bacteria. It has been observed that all the essential oils possess both bacterio-static and bactericidal activity much higher than that of synthetic antibiotics when tested *in vitro*. These essential oils may be effective on other Gram-ve and Gram+ve bacteria. More importantly, these can be included in the list of herbal medicines due to their high antimicrobial potential and lesser side effects. Hence, essential oils and their components can be recommended for therapeutic purposes and be used as an alternative medicine.

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